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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/705, 16/28, C12N 5/10, G01N 33/68, C12Q 1/68

A1

(11) International Publication Number: WO 95/06724

(43) International Publication Date: 9 March 1995 (09.03.95)

(21) International Application Number: PCT/GB94/01892

(22) International Filing Date: 1 September 1994 (01.09.94)

(30) Priority Data:
9318105.5 1 September 1993 (01.09.93) GB

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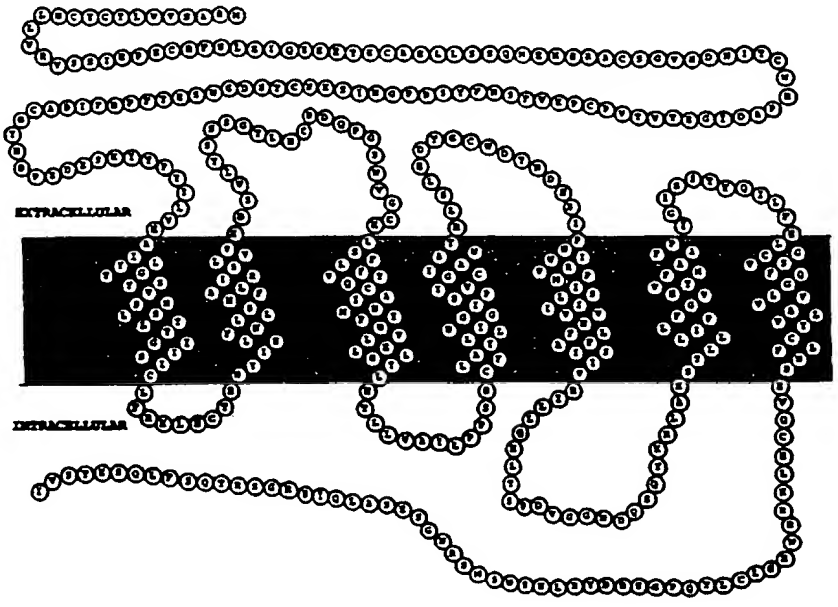
(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: VIP_2 (VASOACTIVE INTESTINAL POLYPEPTIDE) RECEPTOR



(57) Abstract

The present invention provides a VIP_2 receptor gene (seq ID No.1), and polynucleotide probes specifically binding to this gene or to naturally occurring variants thereof, as well as polypeptide probes specifically binding to the receptor polypeptide or to naturally occurring variants thereof. Expression of the gene in a host is also useful for providing means for use in the evaluation of VIP_2 agonists and antagonists.

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VIP2 (VASOACTIVE INTESTINAL POLYPEPTIDE) RECEPTOR

Background of invention

Field of invention

This invention relates to the discovery of cDNA sequences encoding for a newly found member of the recently discovered secretin family of G-protein linked neuropeptide-receptors, which is a receptor for vasoactive intestinal polypeptide (VIP), and the application of this discovery to inter alia 1) the development and use of nucleotide sequences derived from the receptor for the measurement (qualitative and quantitative) and regulation of gene expression and 2) the development and use of VIP agonist and antagonist analogues for clinical and therapeutic use in the management of human pituitary dysfunction, various cancers of the brain, pituitary, lung, adrenal, and reproductive system and VIP related disorders of brain function, and other conditions involving VIP.

Description of the Prior art

Vasoactive intestinal polypeptide (VIP) has been identified and purified from small intestine on the basis of its potent vasodilator activity[1, 2]. VIP has a number of actions in the periphery including vasodilatation, stimulation of electrolyte secretion and smooth muscle relaxation[3]. VIP is a member of a family of structurally related polypeptide hormones which include glucagon, glucagon-like peptide I (GLP), peptide histidine isoleucine (PHI), secretin, pituitary adenylate cyclase activating polypeptide (PACAP) and growth hormone releasing hormone (GHRH). All of these peptides are thought to exert their actions through G-protein linked membrane receptors coupled to adenylate cyclase. Recently, a receptor for VIP[4], has been cloned from rat lung[5] and from a human colon carcinoma cell line[4]. Receptors for glucagon[6-8], GLP[9], GHRH[10-12], secretin[13] and PACAP[14-16] have also been cloned.

Together with receptors for calcitonin[17, 18] and parathyroid hormone (PTH)[19, 20] they form a protein family distinct from other G protein-linked receptors.

VIP has been shown to bind with high affinity to VIP receptors in a variety of tissues. These binding sites are present in lung, liver and intestine, as well as several regions of the brain (e.g. cerebral cortex, hypothalamus and hippocampus[21, 22]). This receptor is probably identical to a receptor known as the PACAP Type II receptor[23, 24] which recognises VIP, PACAP-27 and PACAP-38 with very similar affinities and may correspond to the previously cloned VIP receptor[4, 5] which has a similar tissue distribution[5]. This receptor is present in a variety of peripheral tissues including rat liver, rat lung, mouse splenocytes and human small intestinal epithelium[23, 25-28]. There is some evidence for the existence of other types of VIP receptor in the brain and periphery[29]. Cross-linking studies have been used to identify VIP binding proteins in tissues with molecular weights ranging from 46,000 to 73,000 depending on the tissue and species[30]. Studies with peptide analogs also suggest the existence of more than one pharmacologically distinct class of VIP receptor[31-35]. A class of VIP receptor, for which helodermin is the most potent ligand, has been identified in certain human cell lines, including the lymphoblastic cell line SUP-T1[36, 37] the THP-1 monocyte/macrophage cell line[38] and NCI-H345 lung carcinoma cells[39]. Hill et al. [40] have distinguished two subtypes (or different functional states of a single subtype) of VIP receptor which differ in their sensitivity to GTP analogs. In some brain regions, guanylyl-imidodiphosphate (GMPPNP) substantially inhibited VIP binding. In other regions, VIP binding was insensitive to GMPPNP. Both types of receptor are present in the mouse embryo[41], where they are differentially regulated by treatment with a VIP antagonist.

To date, however, there have been no reports of the sequencing of any gene encoding a second VIP receptor with a distinct and different molecular structure.

The initial step in VIP action centres on the binding of VIP to a specific membrane bound receptor. Multiple steps are then involved in triggering the synthesis of intracellular cAMP (cyclic Adenosine Monophosphate) in tissues such as brain, pituitary, lung, pancreas, gastrointestinal tract, kidney, reproductive tract, blood vessels and various others and in certain tumours. In the central nervous system, VIP may play a role in the pathogenesis of psychiatric, neurological and neuroendocrine disorders. VIP may also regulate cerebral energy metabolism[42] and neuronal survival[43]. VIP stimulates prolactin secretion from the pituitary[44], catecholamine release from the adrenal medulla[45] and in the immune system it inhibits mitogen activated proliferation of T cells by inhibiting interleukin-2 production[46].

Knowledge of the structure and characteristics of VIP receptors in the brain and pituitary and other tissues would also be helpful both in attempting to use VIP or its analogs in pharmacotherapy and in attempting to understand the possible roles of VIP in the body.

Thus there is a particular need for providing probes and other assay materials and methods for use in the detection and/or identification, directly or indirectly, of abnormalities in VIP receptors, especially human VIP receptors and/or their expression, and/or the genes encoding them, as well as means for use in the evaluation of potential VIP agonists or antagonists for use in the treatment of such abnormalities.

It is an object of the present invention to avoid or minimise one or more of the above problems or disadvantages.

Summary of Invention

We have now succeeded in the cloning and expression of a specific, adenylate cyclase-linked VIP receptor from rat brain. We have named this receptor the VIP_2 receptor to distinguish it from the receptors cloned from rat by Ishihara et. al. [5] and from human by Sreedharan et. al. [4]. The latter two receptors probably represent species variants of one gene, which we will refer to as the VIP_1 receptor. The VIP_2 receptor was identified by PCR of rat pituitary cDNA using degenerate oligonucleotide primers corresponding to the third and seventh transmembrane domains of the secretin family of G-protein linked receptors. Full length cDNAs were isolated from an olfactory bulb cDNA library. The cDNA sequence for the novel VIP receptor is presented as the basis of this invention. The sequence is presented in interleaved format (see Fig. 1), and has been submitted to the EMBL/Gen Bank database under accession No. Z25885.

Thus we have shown for the first time that VIP receptor occurs naturally in at least two different forms with distinct tissue distributions and different molecular structures. This indicates the possibility of different physiological functions for the different receptors and the need for diagnostic means and methods for distinguishing these different forms, as well as the possibility of providing VIP2 agonists and antagonists adapted for selective interaction with the newly discovered VIP_2 receptor, for example for the purposes of controlling blood pressure and/or renal function, as well as certain tumours. Given the co-existence of both VIP_1 and VIP_2, provision by means of the present invention of cloned VIP_2 receptor gene and systems for expression thereof, is particularly valuable in providing means for assaying VIP_2 - specific agonists and antagonists.

In more detail, the present invention provides: a gene encoding VIP_2 receptor; and preferably a gene encoding VIP_2 receptor having substantially the amino acid SEQ ID No:1 disclosed herewith and includes genes encoding VIP receptor which genes have substantial nucleotide sequence homology with the nucleotide sequence SEQ ID No:1 disclosed herein; and in particular the genes of the present invention in a form substantially free from other genes. It will be understood that the genes of the present invention may include nucleic acid sequences (upstream and/or downstream of the receptor coding sequence) which are utilized in the expression of the gene such as promoter, operator, and terminator sequences as well as other sequences which do not inhibit its expression. Thus the expression "gene" includes DNA (including cDNA) and/or RNA sequences as well as plasmid or viral "genes" containing the receptor gene and expression vectors for the gene.

With respect to the distribution of VIP2 receptor in the body we have found that significant, and in some cases particularly high, concentrations are to be found in inter alia: the supra-chiasmatic nucleus of the brain which is involved in the control of circadian (or diurnal) and other biorhythms including those involved with sleep and arousal, feeding, drinking, various endocrine functions, and ovulation; the dorsal horn of the spinal cord and especially the substantia gelatinosa and Clark's column which are involved in the transmission and processing of pain; and tumours, especially gastro-intestinal and lung tumours.

In one aspect therefore the present invention provides new methods and means based upon the newly discovered VIP_2 receptor nucleotide sequence for use in the clinical diagnosis and therapeutic management of those processes and abnormalities thereof that involve the acti n of VIP and th VIP_2 recept r, including but not exclusive of:

Neurological and psychiatric disorders including schizophrenia, depression, Alzheimer's disease and Parkinson's disease; Brain tumours; Pituitary tumours and neuroendocrine disorders; Cardiovascular and inflammatory disorders; Disorders of fertility, control of fertility, testicular tumours; impotence directly related to failure to achieve erection; Disorders of the kidney; Phaeochromocytoma and other tumours of neuroendocrine origin; Lung tumours; Leukaemia, immune and inflammatory disorders; Gastro-intestinal Tumours; Pancreatic tumours, pancreatitis, cystic fibrosis; Diabetes; Disorders of foetal development; especially foetal brain development; and Degenerative disorders of the nervous system.

In addition the invention provides methods and means for use in the control and/or regulation of circadian (or diurnal) and other biorhythms including those involved with sleep and arousal, feeding, drinking, various endocine functions, and ovulation; as well as of pain thereby providing valuable new alternative means for providing analgesia.

In the case of tumours there may be used anti-sense polynucleotides in order to restrict or inhibit the growth of tumours. Preferably there are used anti-sense polynucleotides capable of binding to part of the gene which includes the ATG (initiation) codon corresponding to the start of the VIP2 protien coding region.

In general the VIP2 receptor gene sequences are also useful for the design of oligonucleotide probes capable of specifically hybridising with the genes of the present invention, and for the synthesis of polypeptides which may be used in immunoassays. In addition parts of the cDNA sequence may be used to design and/or provide oligonucleotide probes for use in identifying human and other mammalian VIP receptor genes. Thus the present invention also specifically extends to a human gene DNA

sequence which has been obtained directly or indirectly through recovery of a human gene by hydridisation thereof with a polynucleotide probe comprising a labelled DNA or RNA sequence capable of specifically hybridizing to a gene encoding VIP2 receptor having the amino acid sequence of SEQ ID No. 1 disclosed herein. In general any part of the VIP2 receptor coding region of the gene may be used for this probe. Both oligonucleotide probes and the polypeptides may be useful for the diagnosis of VIP2 receptor abnormalities. (It will be understood that references to oligonucleotide probes and the use thereof also include such probes as part of longer sequences). Polypeptides encoded within the cDNA sequences may also be used to raise antibodies against selected regions of normal or abnormal VIP2 receptor polypeptide, which are particularly implicated in ligand binding i.e. the first, second, third and fourth extracellular domains of the VIP2 polypeptide (see fig 2); or in signal transduction i.e. the first, second, third and fourth intra-cellular domains, especially the main cytoplasmic loop or third intracellular domain, most preferably in the region of the interface between the third intracellular domain and the sixth transmembrane region. e.g. one of the four extra-cellular domains identified hereinbelow, and for the purification of antibodies directed against such regions. These antibodies may be useful in immunoassays for detecting normal or abnormal VIP_2 receptor in individuals.

Furthermore the invention provides screening means for use in the evaluation of new VIP_2 receptor agonists and antagonists, comprising a cell transformed with a recombinant expression system comprising an open reading frame (ORF) of DNA derived from a VIP_2 receptor gene or VIP_2 receptor cDNA, said ORF being operably linked to a control sequence compatible with said cell, as well as such expression systems per se.

Thus the present invention further includes a method of producing receptor which method includes the step of expressing the genes of the present invention in a host, as well as VIP_2 receptor produced by such a method. Various suitable hosts are known in the art though eukaryotic hosts are generally preferred, e.g. Xenopus oocytes and COS-7 cells. Prokaryotic hosts that may be used include E. coli. and B. Subtilis. Fungi e.g. yeast may also be used.
For the purposes of developing VIP_2 agonist and antagonist analogues, it is particularly preferred to use cloned cell lines expressing human VIP_2 receptor. Genes for human VIP_2 receptor can be isolated and sequenced by screening a human cDNA library preferably a human tumour (e.g. adrenal, pancreas, lung or brain) or normal adult or foetal human brain, with the aid of oligonucleotide probes from the entire rat VIP2 receptor sequence or parts thereof.

Thus the present invention also includes products and processes utilizing, directly or indirectly, human VIP_2 receptor sequences obtained in this way.
In particular the invention provides means for the evaluation of new VIP_2 receptor agonists and antogonists. These may employ expression systems of the invention directly or in some cases simply the membranes (with VIP_2 receptor) thereof. Conveniently the transfected or transformed cells, or the membranes thereof, are exposed to a suitable ligand e.g. labelled VIP, and the potential agonist or antagonist and the effect of the latter on the binding of the ligand is monitored, e.g. by examining the variation of binding level with agonist/antagonist doseage. Alternatively or additionally there may be monitored a natural or artificial intra-cellular messenger system such as cAMP production, inositol phosphate turnover, cell calcium concentration, or expression of an artificial gene containing an enzyme marker producing a colour or light reaction etc.

A suitable method of restriction enzyme analysis in this invention depends on Restriction Fragment Length Polymorphisms (RFLPs). A sample is taken from any suitable tissue such as blood. DNA is extracted from the cells in any conventional way. It is then digested with an appropriate restriction enzyme e.g. one which cuts in CG-rich sequence. The fragments of different length are separated by gel electrophoresis in any conventional way. A restriction fragment pattern is generated. Probing of the fragments will generally be necessary for clearer detection of the pattern and of the fragment(s) of interest, e.g. a fragment which extends from restriction sites "n" to "n + 2" (where "n" denotes any arbitrary number), seemingly not being restricted at the normal site "n + 1" lying between "n" and "n + 2" due to an abnormality at the "normal" restriction site. Alternatively, a polymorphism might generate restriction enzyme sites and thereby given rise to a plurality of shorter fragments where the normal DNA provides longer ones. Whether it is appropriate to probe for long or short fragments will therefore depend on the circumstances of the polymorphism. In some instances, the probe will extend outside the region designated.

Although the RFLP method is one method of assay, it cannot be ruled out that direct hybridisation of probes to the genomic region will be of interest. Thus suitable biopsy or other samples can be subjected to cloning techniques, to isolate a library of genomic DNA, or PCR of genomic DNA or cDNA. Clones containing the gene can be amplified by Polymerase Chain Reaction (PCR) and probes complementary to the said region used directly on PCR products, which need not be first restricted by enzymes.

It will be appreciated, therefore, that the cDNA of the invention also has uses in assays which are not of the RFLP type. Accordingly, the polynucleotides per se are part of this invention, as 'intermediates' suitable (when labelled)

for use as probes. Both double-stranded and single-stranded polynucleotides are included as well as sense and anti-sense forms. Suitable polynucleotide probes may be oligonucleotides of from 10 to 50, preferably from 16 to 30 nucleotides in length. Shorter probes are unlikely to be sufficiently specific for the sequence of interest. Longer polynucleotide probes of from 100 to 500 nucleotides or more may also be used with longer ones (up to 2000 or more nucleotides) being useful e.g. for chromosmal in-situ hybridisation as further described hereinbelow. Preferably the probes relate to parts of the polynucleotide sequence corresponding to one or more domains, or portions thereof, of the VIP2 polypeptide, which are particularly implicated in ligand binding i.e. the first, second, third and fourth extraceullular domains of the VIP2 polypeptide (see Figs.1 and 2); or in signal transduction i.e. the first, second, third and fourth intra-cellular domains, especially the main cytoplasmic loop or third intra-cellular domain, most preferably in the region of the interface between the third intracellular domain and the sixth transmembrane region. The probe will usually be of DNA or RNA and labelled in any suitable manner e.g. by labelling with an enzyme, radioisotope, fluorescent, luminescent, or chemiluminescent labels or biotinylation.

The fragments are probed under any appropriate conventional hybridisation conditions, the fragments being conveniently first transferred to a filter. The complexes thus formed are detected by autoradiography or other detection means appropriate to the particular kind of label used.

Abnormalities in the polynucleotide sequence of restriction fragments of the genomic DNA or cDNA coding for VIP_2 receptor which are as small as single-point mutations can also be detected by means of Temperature Gradient Gel Electroph resis in which a temperature gradient is superimposed, parallel to or transversely of, the electrical

field in gel electrophoresis. The method is based on the fact that the temperature of denaturation of double stranded (ds) DNA is altered by changes in polynucleotide sequence. Furthermore, partial denaturation of a DNA duplex causes a change in electrophoretic mobility. Further details of this technique are described in the literature by Reisner et al, 1989 (56) and, Birmse et al, 1990 (57).

We have now further found that the chromosmal location for the VIP2 receptor gene is at 7q36.3 and thus the present invention now also provides further diagnostic means for use in the detection of conditions associated with VIP_2 abnormalities, which comprises one or more of hybridisation of a polynucleotide probe of the invention with the 7q36.3 chromosome region; and examination of the 7q36.3 chromosome region for gross abnormalities. Major defects in the 7q36.3 chromosomal region have been found to be associated with the birth defect holoprosencephaly, especially the type 3 form, and thus the present invention also provides a method of diagnosis for holoprosencephaly comprising the steps of collecting a foetal sample and examining the chromosomal content thereof by one or more of the techniques described hereinbefore so as to detect the presence of defects or irregularities in the 7q36.3 chromosomal region.

The VIP_2 receptor cDNA cloned and sequenced is shown in Fig. 1A. Fig. 2 illustrates schematically the predicted transmembrane, intracellular and extracellular domains of the receptor molecule. The transmembrane domains consist of seven stretches of hydrophobic in nature amino acids which span the membrane. The extracellular domain consists of four hydrophilic in nature amino acid stretches which exist exterior to the cell membrane. This region is believed to be important for the recognition of specific ligands. The intracellular domain consists of four hydrophilic stretches of amino acids which are thought to be involved in signal transducti n.

It will be appreciated that abnormality in human VIP_2 receptor and/or its expression, may be "assayed" in a number of ways. Thus the DNA encoding the VIP_2 receptor may itself be assayed for the presence or absence of abnormalities or the VIP_2 receptor polypeptide may be assayed for such purposes, where this is actually expressed.

The former case generally involves the use of labelled polynucleotide probes to hybridise with DNA within the region coding for VIP_2 receptor polypeptide for the purposes of indicating the presence of absence of particular polynucleotide sequences. In the latter case antibody probes are used to form antigen-antibody complexes with regions of the expressed polypeptide for the purposes of indicating the presence or absence of particular polypeptide sequences. It will be understood that once more or less common or typical abnormalities have been specifically identified e.g. by initially probing with 'normal' polynucleotide and then sequencing, polynucleotide probes can be synthesized or otherwise produced with sequences corresponding to or complementary to the "abnormal" sequences, to allow screening of tissue samples for specific VIP_2 receptor gene abnormalities. In the case of gene abnormalities such as the complete or substantial absence of the VIP_2 receptor gene, then this may be detected by failure to yield any binding with suitable probes or absence of any product from PCR amplification using polynucleotide probes complementary to substantially spaced apart portions of the gene.

In the case of assays of the polypeptide itself, suitable stretches of amino acids based on the cDNA sequence information provided by the present information, may be synthesised on a peptide synthesiser. These peptides would generally have a length of from 10 to 50, preferably 15 to 30, amino acids but could be even shorter or longer. Alternatively the complete VIP_2 recept r polypeptide or fragments thereof may be expressed in a suitable eukary tic

or prokaryotic host such as E. Coli using an appropriate vector. Polyclonal antibodies to these peptides may be produced by conventional approaches such as the immunisation of host animals (rabbit, goat etc.) with said peptides, optionally conjugated to a protein carrier such as thyroglobulin, and recovery of the desired antibody material therefrom. Monoclonal antibodies could also be raised using conventional monoclonal antibody-production procedures.

It will also be understood that "assay" may be either qualitative or quantitative (e.g. where detection of under or over-expression of the receptor is required).
Further preferred aspects of the invention are indicated in the following patent claims.

Detailed Description

Isolation and Sequencing of VIP_2 receptor Gene

Preparation of Rat Pituitary cDNA

Anterior pituitary glands from male rats (Cob Wistar, 250g) were removed and total RNA was isolated by the method of Chomczynski and Sacchi[47]. Single stranded cDNA synthesis and PCR were carried out using a commercial kit (Perkin Elmer Cetus). 1μg RNA was annealed with 2.5μM random hexanucleotide primers by heating to 95°C for five minutes, then cooling to 4°C over fifteen minutes. Single stranded cDNA was synthesised by incubating the oligonucleotide-RNA solution at 42°C for 15 minutes in 20μl 10mM Tris-HCl, pH 8.3, containing 50mM KCl, 5mM MgC12, 1mM each dNTP, 20 units RNase inhibitor and 50 units reverse transcriptase. The reaction was terminated by heating to 99°C for 5 minutes.

Selective Amplification of Rat Pituitary cDNA

PCR was performed using a pair of degenerate 32-mer oligonucleotide primers (Figure 3), corresponding to c nserved regions in the third and seventh transmembrane domains of the rat secretin[13], pig calcitonin[18] and

opossum parathyroid hormone PTH[19] receptors. In more detail, each "primer" comprises a mixture of a large number of oligonucleotides with different permutations of nucleotide at particular positions within the oligonucleotide sequence corresponding to the codons for particular amino acids within the receptor peptide sequence coded for thereby. This is necessary in order to include different codons which code for the same amino acid due to the degeneracy of the genetic code. Alternative nucleotides are indicated above each other. Where it is desired to include the possibility of any one of the A, G, C, and T nucleotides (containing the bases Adenine, Guanine, Cytidine, and Thymidine) then the I nucleotide (with the base Inosine) is used. Sequences containing restriction sites (EcoRI and BamHI, respectively) were added to the ends of the two oligonucleotide primers. Sequences of the two oligonucleotides are shown, aligned with the corresponding amino acid sequences in the rat secretin, pig calcitonin and opossum PTH receptors in Fig.3.

Reactions (100µl) contained 30pmol of each primer, 20µl reverse transcriptase reaction and 2.5 units Amplitaq DNA polymerase in 50mM KCl, 10mM Tris-HCl, pH 8.3 and 2mM MgCl25 cycles of PCR (60s denaturation at 94°C, 60s annealing at 45°C, 60s extension at 60°C) were followed by a further 40 cycles (60s denaturation at 94°C, 60s + 6s per cycle annealing and extension at 60°C) followed by 7 minutes at 60°C. PCR products were precipitated with ammonium acetate and ethanol after which one half of each reaction was run on a 1% agarose gel. Five bands containing cDNA sequences having a size corresponding generally to that expected of the cDNA within the receptor gene between the two primers used, and ranging in size from 500bp to 900bp were excised from the gel and purified using the Sephaglas BandPrep kit (Pharmacia) and one quarter was used for a further round of PCR (5 cycles of 60s denaturation at 94°C, 60s annealing at 47°C, 60s extension at 60°C foll wed by a

further 40 cycles of 60s denaturation at 94°C, 60s + 6s per cycle annealing and extension at 60°C follow d by 7 minutes at 60°C.

Preparation of cDNA clones

PCR products were ethanol precipitated, digested with BamHI and EcoRI, separated on a 1% agarose/TAE gel, purified using the Sephaglas BandPrep kit (Pharmacia), ligated into pBluescript SK-Vector (Stratagene) and used to transform competent E. coli DS941. The sequences of several of the clones were obtained on both strands (Sequenase 2.0 kit, USB). One of the clones (RPR4) was found to have substantial similarity to rat VIP_1 receptor[4, 5] but was distinct from all the known members of the family.

Tissue Distribution of mRNA (Northern Blotting)

Total RNA was isolated from tissues using the guanidinium thiocyanate/caesium chloride method[48]. Approximately 20µg of each RNA was separated by electrophoresis on denaturing 1% agarose/formaldehyde gels, transferred to a nitrocellulose membrane (Hybond-C, Amersham) and baked for 2h at 80°C. The membrane was then hybridised with the insert from RPR4 that had been labelled with [32P]dCTP-using random hexanucleotide primers (Pharmacia) and the Klenow fragment of E.*coli* DNA polymerase[49]. Hybridisation was performed overnight in 50% formamide, 25mM KP04, pH7.4, 5xSSC (1xSSC=0.15M NaCl, 0.015 M Na citrate pH7.0), 5x Denhardt's solution, 50µg/ml salmon sperm DNA. They were then washed twice for 20 min in 2xSSC/0.1%5DS at 50°C, then twice more in 0.5xSSC/0.1%5DS at 50°C and exposed to Fuji RX film. This Northern blot analysis using RPR4 as a hybridisation probe revealed a mRNA transcript of approximately 3.5 kilobases expressed in the pituitary and regions of the brain, with the highest levels being observed in the olfactory bulb.

Screening Rat Olfactory Bulb cDNA Library

One of the clones isolated, RPR4, was used accordingly to screen a commercial cDNA library in the Lambda Zap II vector[50] prepared from rat olfactory bulb (Stratagene Catalogue No. 936520).

$4x10^5$ plaque forming units of the library (corresponding to one-fifth of the total number of $2x10^6$ primary plaques) were plated onto 20x 140mm plates using *E.coli* XL1-blue as a host strain. Filters containing plaque lifts from these plates were denatured by submerging in 1.5M NaCl, 0.5M NaOH for 2 mins, followed by neutralization in 1.5M NaCl, 0.5M TrisHCl pH8.0 for 5 mins, then rinsed in 3xSSC. The filters were then blotted dry on Whatman 3MM filter paper and baked at 80°C for 2 hours. They were then hybridised with the insert from RPR4 labelled using the same method found in the Northern Blot protocol as described hereinabove. Hybridisation was performed overnight in 50% formamide, 6x SSPE, 5x Denhardt's, 0.5% SDS, 100µg/ml salmon sperm DNA at 45°C. Positive plaques were identified, picked, and the phage purified by further rounds of plating and screening. This procedure yielded six positive clones, of which three were analysed further. Positive clones were excised by co-infecting E.*coli* strain XL-1 blue with ExAssist (TM) helper phage (Stratagene) Bluescript SK phagemids (packed as filamentousphage particles) were then used to generate double-stranded plasmids by infecting E.*coli* strain SOLRTM, and plating on L-broth/ampicillin plates to produce colonies. The pBluescript SK-double-stranded plasmid was then recovered using standard techniques[51].

Three different clones were isolated and characterised by restriction mapping and sequencing. All three had sequences corresponding to RPR4 but only one cDNA, RPR4/6.3 containing an insert of 3.3 kb, encoded a complete open reading frame, encoding a protein of 437 amino acids with a predicted molecular weight of 49519 (Figure 2). A 22 amino acid hydrophobic signal sequence is found at the amino-terminal

end, with a predicted signal cleavage site between Pro^{22} and Glu^{23}[52]. A hydropathy plot shows seven hydrophobic, putative membrane-spanning domains (Figure 2). Comparison with other members of the secretin/calcitonin/PTH receptor family (Figure 3) revealed that the predicted protein encoded by RPR4/6.3 has greatest similarity with the rat VIP_1 and PACAP type I receptors (50% identity, with each). The highest amino acid sequence identity is found in the putative transmembrane regions, whereas the sequences of the amino-terminal extracellular domains and the carboxyl-terminal cytoplasmic ends are highly divergent.

Expression of VIP_2 receptor Gene

In order to determine the pharmacological characteristics of the novel receptor, the insert from the full length clone (RPR4/6.3) was excised as an EcoRI fragment and ligated into the EcoRI site of the mammalian cell expression vector pcDNA-1 (Invitrogen)[53, 54], and transiently transfected into COS-7 cells.

COS 7 cells were grown in DMEM supplemented with 10% new-born calf serum and 100U/ml each of penicillin and streptomycin, in a humidified atmosphere of 95% air/ 5% CO_2 at a constant temperature of 37°C, and were passaged every 3-4 days. Cells for transfection were trypsinised the day before the experiment and plated at a density of approximately 40-50% confluency in 75cm2 flasks.

For transfection, cells were washed twice with OptiMEM (Gibco) supplemented with 100U/ml each of streptomycin and penicillin at 37°C before exposure to transfecting medium for 4h. The transfecting medium consisted of OptiMEM/ penicillin/ streptomycin, 400μg/ml DEAE dextran (Promega), 100μM chloroquine phosphate (Sigma) and 10-20μg plasmid per flask. This was replaced with 10% DMSO in PBS for 2 min, then DMEM/ 2% UltraSer G/ penicillin/ streptomycin. Cells

were grown for 24h, then trypsinised and re-plated. Cells were harvested 48h later.

Functional Assay of VIP_2 receptor Gene

Since all members of the secretin receptor family so far identified are associated with activation of adenylate cyclase (also referred to in the art as adenylyl cyclase) the transfected cells were stimulated with several potential ligands, and intracellular cAMP levels measured by radioimmunoassay.

For screening purposes, cells transfected with RPR4/6.3 were seeded onto 12-well tissue culture dishes, and for dose/response experiments, onto 24-well dishes. Prior to incubation with various peptide ligands, the cultures were washed with DMEM containing 0.25% BSA, and pre-incubated at 37 C for 30 min in the presence of 0.5mM isobutyl methylxanthine (IBMX). VIP ligand and other peptides were directly added at concentrations indicated in Figs. 4a and 4b and incubated at 37 C for 15-30 min. The reaction was stopped by adding ice cold 0.1M HCl, and the cells homogenised by trituration. The levels of cAMP in the acidic extracts were measured by radioimmunoassay using antiserum cAB4 (courtesy of K.J. Catt, NICHD, NIH, Bethesda, MD)[55].

Results

Fig. 4a shows the effect of stimulating cells transfected with RPR4/6.3 by various peptide ligands. Values of cAMP release are expressed (mean ± SEM, n=3) as a percentage of the stimulation evoked by 100nM VIP. While treatment with corticotropin releasing factor (CRF), calcitonin gene related peptide (CGRP), secretin and glucagon, exhibited negligible effect, treatment with VIP, PACAP27, and PACAP-38 resulted in a marked elevation of cAMP levels. PHI and rat GHRH (rGHRH) also stimulated cAMP levels but were significantly less potent. PHI, VIP and PACAP-38 failed to

stimulate cAMP levels in a control experiment where COS-7 c lls had been transfected with the $5HT_{1A}$ (Serotonin) receptor (data not shown).

As shown in Fig.4b the stimulation of cAMP levels by VIP, PACAP, helodermin, and PHI was dose-dependent. The EC50 (peptide concentration for 50% of maximal effect) for cAMP accumulation was approximately 0.18nM for PACAP-38, 0.43nM for PACAP-27, 0.25nM for helodermin, 0.17nM for VIP and 2.14nM for PHI. (The values shown in Fig.4b are the mean of 3. Basal cAMP was 0.8 ± 0.15 pmoles/well). The maximal stimulation of cAMP accumulation by rGHRH (400nM) was only 60% of that found for VIP. We therefore conclude that the order of potency of these ligands is VIP~ PACAP38~ PACAP27~ helodermin >PHI >>rGHRH, and that accordingly, RPR4/6.3 encodes a high affinity receptor for VIP.

Description of the Figures

Fig. 1 shows the gene encoding the rat VIP_2 receptor. The polynucleotide sequences specifically elucidated thus far are indicated along with the deduced amino-acid sequence;

Fig. 2 shows schematically the 7 transmembrane domains of the rat VIP_2 receptor and the 4 extracellular and 4 intracellular domains of the amino acid sequence derived from RPR4/6.3 using standard single letter codes to represent the amino acids;

Fig.3 shows the sequences for the two degenerate oligonucleotide primers; and

Fig.4 shows cAMP accumulation monitored during functional assay of a cloned gene of the invention.

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CLAIMS

1. A gene encoding VIP_2 receptor.

2. A gene encoding VIP_2 receptor having the amino acid sequence of SEQ ID No.1.

3. An isolated DNA sequence encoding VIP_2 receptor.

4. A recombinant DNA sequence encoding VIP_2 receptor.

5. An isolated or recombinant DNA sequence for use in expression in a prokaryotic or eukaryotic host cell of a polypeptide product having an amino acid sequence corresponding to a sufficient extent to that of a naturally occurring VIP_2 receptor to provide at least one biological function of said naturally occuring VIP_2 receptor, which DNA sequence is selected from:

a) the DNA sequence of SEQ ID No. 1 or a sequence complementary thereto; and

b) a DNA sequence which can hybridize to a DNA sequence defined in (a) or to a fragment thereof.

6. A gene or sequence according to any one of claims 1,3,4 and 5b wherein said VIP_2 receptor is human VIP_2 receptor.

7. A gene or sequence according to claim 6 which has been obtained directly or indirectly through recovery of a human gene by hydridisation thereof with a polynucleotide probe comprising a labelled DNA or RNA sequence capable of specifically hybridizing to a gene according to claim 2.

8. A prokaryotic or eukaryotic host cell transformed or transfected with a gene or DNA sequence according to any one of the preceding claims.

9. A recombinant vector containing a DNA sequence or gene according to any one of claims 1 to 7.

10. A prokaryotic or eukaryotic host cell transformed or transfected with a recombinant vector according to claim 9.

11. A polynucleotide probe comprising a labelled DNA or RNA sequence capable of specifically hybridizing to a gene according to claim 1, claim 2 or claim 6 when dependent on claim 1 or claim 2, or a naturally occurring variant thereof.

12. A method of detecting a gene according to claim 1 or claim 2 or claim 6 when dependent on claim 1 or claim 2, which method comprises hybridizing a probe according to claim 11 with said gene, and detecting bound labelled probe.

13. An antibody probe comprising a labelled antibody raised against an amino acid sequence capable of specifically binding to VIP_2 receptor expressed by a gene according to claim 1 or claim 2 or claim 6 when dependent on claim 1 or claim 2, or a naturally occuring variant thereof.

14. An antibody probe according to claim 13 which is a monoclonal antibody.

15. A method of detecting VIP_2 receptor expressed by a gene according to claim 1 or claim 2 or claim 6 when dependent on claim 1 or claim 2, or a naturally occurring variant thereof, which method comprises allowing a probe according to claim 13 or claim 14 to bind with said receptor, and detecting bound labelled probe.

16. A method of diagnosis for a condition associated with VIP_2 receptor abnormality which comprises hybridizing a polynucleotide probe according to claim 11 with the 7q36.3

chromosomal region, and monitoring for absence of bound labelled probe.

17. A method according to claim 16 wherein is used the technique of fluorescence in situ hybridisation.

18. A method of diagnosis for a condition associated with VIP_2 receptor abnormality which comprises amplification by polymerase chain reaction using polynucleotide primers from substantially spaced apart portions of a nucleic acid sequence encoding VIP_2 receptor, and monitoring for absence of amplified polynucleotide sequence product.

19. A method of diagnosis for a condition associated with VIP_2 receptor abnormality which comprises microscopic examination of the chromosomal region for gross abnormality.

20. A method of diagnosis for holoprosencephaly comprising the steps of collecting a foetal sample and examining the chromosmal content thereof by a method according to any one of claims 16 to 19.

21. A method of evaluating a potential VIP_2 agonist or antagonist comprising the steps of: providing cell membrane of a VIP_2 receptor expression system comprising a transformed or transfected host cell according to claim 8 or claim 10, which membrane has VIP_2 receptor; exposing said cell membrane to labelled ligand and to said agonist or antagonist; and monitoring at least one of ligand binding and an intra-cellular messenger system.

22. A method according to claim 21 which includes the preliminary step of providing a VIP_2 receptor expression system comprising a transformed or transfected host cell according to claim 8 or claim 10.

FIG.1

SEQ ID NO: 1
SEQUENCE TYPE: Nucleotide with corresponding protein
SEQUENCE LENGTH: 2128 base pairs

STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: cDNA TO mRNA

ORIGINAL SOURCE
ORGANISM: rat
IMMEDIATE EXPERIMENTAL SOURCE
Olfactory bulb

FEATURES
from 69 to 134 bp signal peptide
from 135 to 1382 bp mature peptide
from 237 to 239 bp potential glycosylation site
from 327 to 329 bp potential glycosylation site
from 339 to 341 bp potential glycosylation site
from 447 to 512 bp transmembrane domain 1 (putative)
from 540 to 599 bp transmembrane domain 2 (putative)
from 675 to 746 bp transmembrane domain 3 (putative)
from 783 to 851 bp transmembrane domain 4 (putative)
from 903 to 974 bp transmembrane domain 5
from 1050 to 1109 bp transmembrane domain 6 (putative)
from 1146 to 1205 bp transmembrane domain 7 (putative)

PROPERTIES: VIP2 receptor for vasoactive intestinal peptide (VIP)

```
GCGCTGGGAG  GCCCCGAGCT  GGCGTTACTG  CTGAGGGCGC  CAAGGACCGA  GGCGGCACTG          60

AGCCCAGG ATG AGG GCG TCG GTG GTG CTG ACC TGC TAC TGC TGG TTG CTG GTG          113
         Met Arg Ala Ser Val Val Leu Thr Cys Tyr Cys Trp Leu Leu Val
                  -20                 -15                 -10

CGG GTG AGC AGC ATC CAC CCA GAA TGC CGG TTT CAT CTG GAA ATA CAG GAA           164
Arg Val Ser Ser Ile His Pro Glu Cys Arg Phe His Leu Glu Ile Gln Glu
         -5              -1  1               5                  10

GAG GAG ACA AAA TGT GCA GAG CTG CTA AGC AGC CAA ATG GAG AAT CAC AGA           215
Glu Glu Thr Lys Cys Ala Glu Leu Leu Ser Ser Gln Met Glu Asn His Arg
                 15                  20                  25

GCT TGC AGC GGT GTC TGG GAC AAC ATC ACA TGC TGG CGC CCT GCA GAC ATT           266
Ala Cys Ser Gly Val Trp Asp Asn Ile Thr Cys Trp Arg Pro Ala Asp Ile
         30                  35                  40
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GGG GAA ACT GTC ACA GTG CCC TGC CCC AAA GTG TTC AGC AAT TTC TAC AGC                317
Gly Glu Thr Val Thr Val Pro Cys Pro Lys Val Phe Ser Asn Phe Tyr Ser
 45                  50                  55                  60

AGA CCA GGA AAC ATA AGC AAA AAC TGC ACT AGT GAT GGG TGG TCG GAG ACA                368
Arg Pro Gly Asn Ile Ser Lys Asn Cys Thr Ser Asp Gly Trp Ser Glu Thr
            65                  70                  75

TTT CCG GAT TTC ATA GAT GCG TGT GGC TAC AAC GAC CCC GAG GAT GAG AGT                419
Phe Pro Asp Phe Ile Asp Ala Cys Gly Tyr Asn Asp Pro Glu Asp Glu Ser
     80                  85                  90                  95

AAG ATC ACG TTT TAT ATT CTG GTG AAG GCC ATT TAT ACC TTG GGC TAC AGT                470
Lys Ile Thr Phe Tyr Ile Leu Val Lys Ala Ile Tyr Thr Leu Gly Tyr Ser
                100                 105                 110

GTT TCT CTG ATG TCT CTT ACA ACA GGA AGC ATA ATT ATC TGC CTC TTC AGG                521
Val Ser Leu Met Ser Leu Thr Thr Gly Ser Ile Ile Ile Cys Leu Phe Arg
        115                 120                 125

AAG CTG CAC TGC ACA AGG AAC TAC ATC CAC CTG AAT CTG TTC CTC TCC TTC                572
Lys Leu His Cys Thr Arg Asn Tyr Ile His Leu Asn Leu Phe Leu Ser Phe
130                 135                 140                 145

ATG CTG AGA GCC ATC TCT GTG CTG GTC AAG GAC AGT GTG CTC TAC TCC AGC                623
Met Leu Arg Ala Ile Ser Val Leu Val Lys Asp Ser Val Leu Tyr Ser Ser
            150                 155                 160

TCA GGT ACA CTG CGC TGC CAC GAC CAG CCG GGC TCC TGG GTT GGC TGC AAG                674
Ser Gly Thr Leu Arg Cys His Asp Gln Pro Gly Ser Trp Val Gly Cys Lys
    165                 170                 175                 180

CTC AGC CTG GTA TTC TTC CAG TAC TGT ATC ATG GCG AAC TTC TAC TGG CTT                725
Leu Ser Leu Val Phe Phe Gln Tyr Cys Ile Met Ala Asn Phe Tyr Trp Leu
                185                 190                 195

CTG GTG GAG GGT CTC TAC CTG CAC ACC CTC CTG GTA GCC ATC CTT CCT CCC                776
Leu Val Glu Gly Leu Tyr Leu His Thr Leu Leu Val Ala Ile Leu Pro Pro
        200                 205                 210

AGC AGG TGT TTC CTG GCC TAC CTT CTT ATT GGA TGG GGT ATC CCC AGT GTG                827
Ser Arg Cys Phe Leu Ala Tyr Leu Leu Ile Gly Trp Gly Ile Pro Ser Val
215                 220                 225                 230

TGT ATA GGT GCA TGG ATA GCA ACT CGC CTT TCT TTA GAA GAC ACA GGT TGC                878
Cys Ile Gly Ala Trp Ile Ala Thr Arg Leu Ser Leu Glu Asp Thr Gly Cys
            235                 240                 245

TGG GAC ACG AAC GAC CAC AGC ATC CCC TGG TGG GTC ATT CGG ATG CCC ATT                929
Trp Asp Thr Asn Asp His Ser Ile Pro Trp Trp Val Ile Arg Met Pro Ile
    250                 255                 260                 265

CTA ATT TCT ATT GTA GTC AAC TTT GCC CTC TTC ATC AGC ATT GTA AGG ATC                980
Leu Ile Ser Ile Val Val Asn Phe Ala Leu Phe Ile Ser Ile Val Arg Ile
                270                 275                 280

TTA CTT CAG AAG CTA ACT TCT CCA GAT GTT GGT GGC AAT GAC CAG TCA CAG               1031
Leu Leu Gln Lys Leu Thr Ser Pro Asp Val Gly Gly Asn Asp Gln Ser Gln
        285                 290                 295

TAC AAG AGG CTC GCC AAG TCC ACA CTG CTG CTA ATC CCA CTG TTT GGC GTC               1082
Tyr Lys Arg Leu Ala Lys Ser Thr Leu Leu Leu Ile Pro Leu Phe Gly Val
300                 305                 310                 315
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CAC TAC ATG GTG TTT GCT GCC TTC CCC ATT GGC ATC TCC TCC ACG TAC CAG          1133
His Tyr Met Val Phe Ala Ala Phe Pro Ile Gly Ile Ser Ser Thr Tyr Gln
            320                 325                 330

ATC CTG TTT GAG TTA TGT GTT GGT TCC TTC CAG GGC CTG GTG GTC GCA GTT          1184
Ile Leu Phe Glu Leu Cys Val Gly Ser Phe Gln Gly Leu Val Val Ala Val
    335                 340                 345                 350

CTA TAT TGC TTT CTG AAC AGT GAG GTA CAG TGT GAA CTG AAA AGA AGG TGG          1235
Leu Tyr Cys Phe Leu Asn Ser Glu Val Gln Cys Glu Leu Lys Arg Arg Trp
                355                 360                 365

AGA GGC CTG TGC CTG ACC CAG CCT GGG AGC CGG GAC TAC CGG CTG CAC AGC          1286
Arg Gly Leu Cys Leu Thr Gln Pro Gly Ser Arg Asp Tyr Arg Leu His Ser
        370                 375                 380

TGG TCC ATG TCC CGG AAT GGC TCA GAA AGC GCC CTA CAG ATA CAC CGT GGC          1337
Trp Ser Met Ser Arg Asn Gly Ser Glu Ser Ala Leu Gln Ile His Arg Gly
385                 390                 395                 400

TCC CGT ACC CAG TCC TTC CTG CAG TCA GAG ACC TCA GTC ATT TAGCTGTGTC          1389
Ser Arg Thr Gln Ser Phe Leu Gln Ser Glu Thr Ser Val Ile
            405                 410

CCTCATACAG  AGCTGACAGT  GCTGCTGGGT  TTGACATATG  TGTTTGCCAG  CTTCCTCTGC      1449
TTGCCCCAGT  GTCTGGTGCC  TTATTGGGTC  AGCCCTGGTC  CTTAACTCGA  TTGTAACTTG      1509
ATTGAAACAC  CAGTTATTGT  TGACAGACTC  TAGCCTTTAA  GCCATCCTCT  TCATAATATG      1569
GCACAGCCAT  ATTCTACTTT  CAAAGAGAGC  AAGGAAACCA  GGTGGCCCTG  AACATCAAAA      1629
CTGGGTTCTA  GAACGTCCGA  AAAAAAACAA  GGAGGAAGAT  TCCAGTTTCT  CCACTGCCTC      1689
CTGTCAGGGA  GAACCACGTC  CAGAACCAGC  TGAGCATCAC  CACCCTGTGG  CCCAGCGCAT      1749
GCCTATGGTT  GTCTCTGACA  TTCCTCTCAA  TCTATGGCAT  ACCAGGGAAA  GTGGATGAAA      1809
GCAACCTTCT  CATCCTACAA  GCTTCCTGGT  GCCCCCGGCC  TTGAGTTCTG  TCCTGTGGAC      1869
TACATCTGGA  CAGCCACACC  CTTGCCATTC  CCTGGACCAT  CTGGTAGTGA  CCAAAGATAA      1929
GTCAGGGAGG  ATATTCATGG  TTCAGCGGCC  AAGCCAGGAA  CTCATCTCCT  CCCAGGGCAC      1989
TTAGCATCTG  CTGCTTCCTA  CAAGTGGTGA  GCAGCCCTGG  ATCCCAGGCC  GGCTGCTGGC      2049
ATGAAGGCCC  TCATTCATGA  CTTGTCCATT  CAGCCCAAGG  TTGTTCTCTA  GCTGGTAGAA      2109
AAACTTCGAT  TTTTATT                                                      2126
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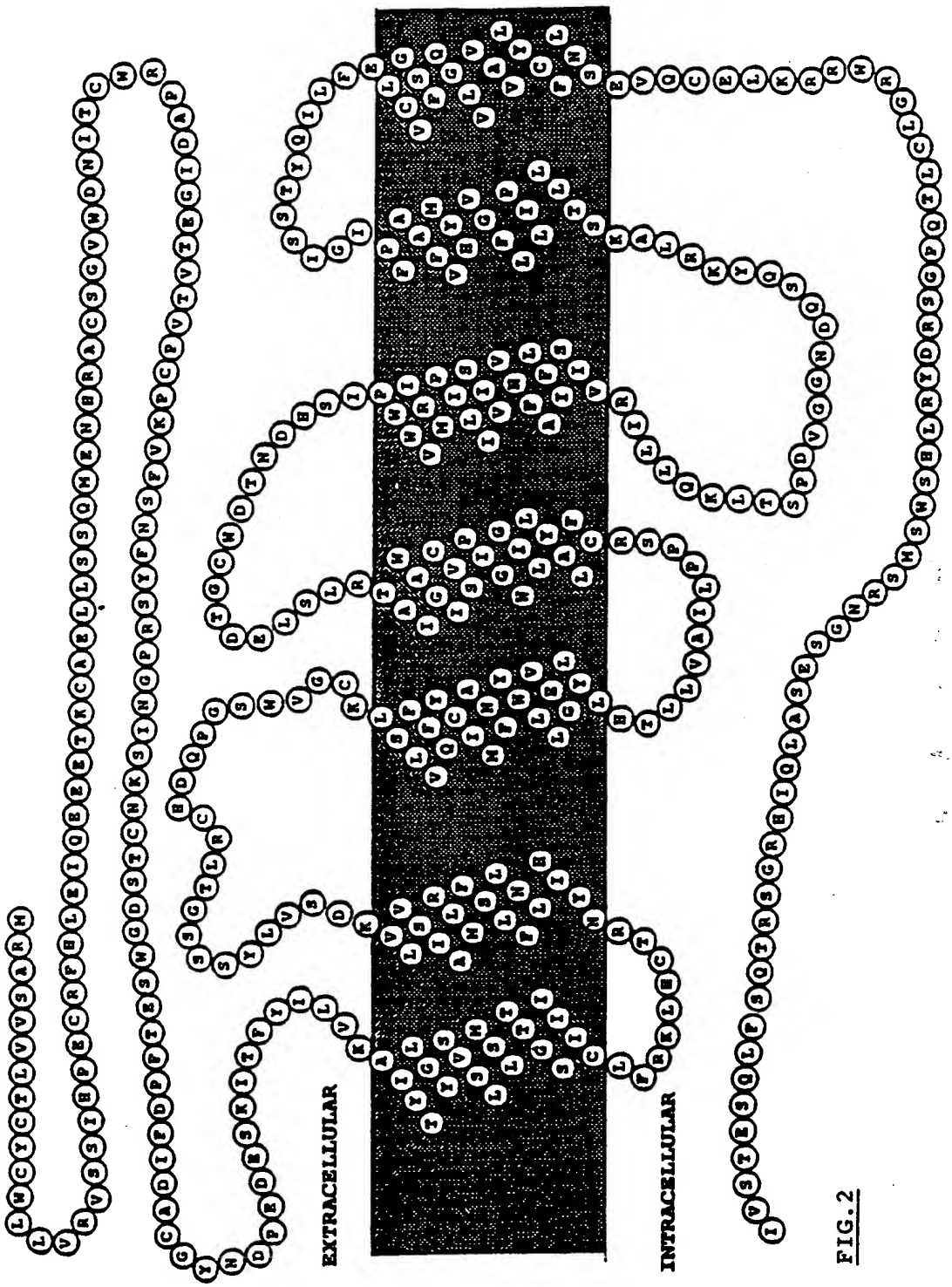

FIG.2

FIG.3

Calcitonin	Asn Tyr Phe Trp Met Leu Cys Glu Gly Val Tyr
Parathyroid hormone	Asn Tyr Tyr Trp Ile Leu Val Glu Gly Leu Tyr
Secretin	Asn Tyr Ala Trp Leu Leu Val Glu Gly Leu Tyr

Oligo 1 5'-GGGAATTC AA$^{C}_{T}$ TA$^{C}_{T}$ G$^{A}_{C}$$^{C}_{T}$I TGG $^{A}_{C}$TI $^{C}_{T}$TI GG$_{TT}$I GA$^{A}_{G}$ GGI $^{C}_{G}$TI TA-3'

(Third Transmembrane)

Calcitonin	Phe Gln Gly Phe Phe Val Ala Ile Ile Tyr Cys
Parathyroid hormone	Phe Gln Gly Phe Phe Val Ala Ile Ile Tyr Cys
Secretin	Phe Gln Gly Leu Val Val Ala Val Leu Tyr Cys

Oligo 2 3'- AA$^{A}_{G}$ GT$^{C}_{T}$ CCI $^{A}_{G}$AI $^{A}_{C}$AI CAI CGI $^{C}_{T}$AI $^{G}_{T}$AI AT$^{A}_{G}$ AC CCTAGGGG-5'

(Seventh Transmembrane)

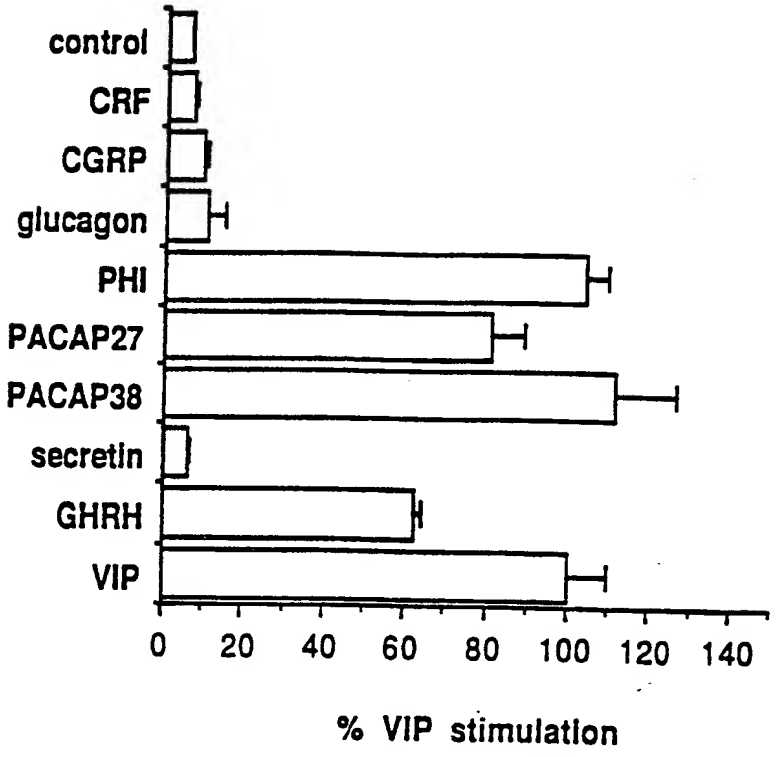
control
CRF
CGRP
glucagon
PHI
PACAP27
PACAP38
secretin
GHRH
VIP
0
20
40
60
80
100
120
140
% VIP stimulation

FIG.4a

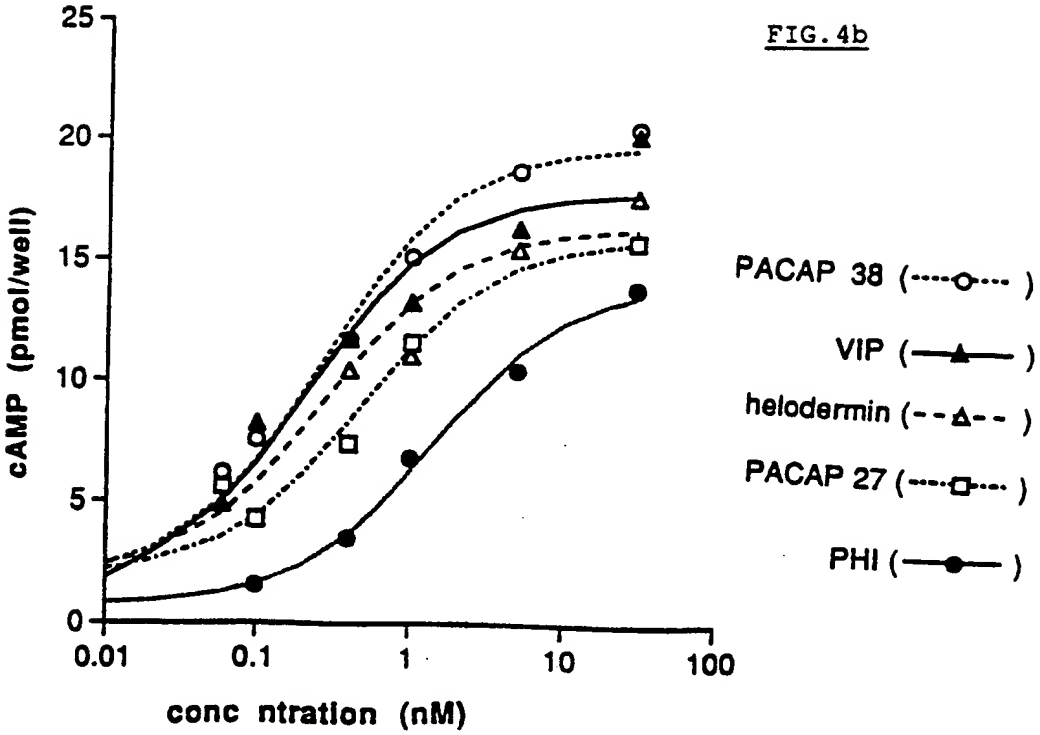
25
20
15
10
5
0
cAMP (pmol/well)
0.01
0.1
1
10
100
conc ntration (nM)
PACAP 38 (----o----)
VIP (—▲—)
helodermin (--△--)
PACAP 27 (----□----)
PHI (—●—)

FIG.4b

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/01892

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 C07K16/28 C12N5/10 G01N33/68
C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	FEBS LETTERS., vol.334, no.1, 8 November 1993, AMSTERDAM NL pages 3 - 8 LUTZ EM;SHEWARD WJ;WEST KM;MORROW JA;FINK G;HARMAR AJ; 'The VIP2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide.' see the whole document ---	1-22
X	WO,A,92 21754 (OSAK BIOSCIENCE KENKYUSHO) 10 December 1922 see figure 1 --- -/--	5-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 1994	30. 12. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Nauche, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE WPI Week 9344, Derwent Publications Ltd., London, GB; AN 93-348480 & JP,A,5 255 394 (ZH OSAKA BIOSCIENCE KENKYUSHO) 5 October 1993 see abstract -----	5-12

INTERNATIONAL SEARCH REPORT

ation on patent family members

Inter[illegible] Application No
PC[illegible] 94/01892

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9221754	10-12-92	JP-A- 4360688	14-12-92